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Toxic threshold of dietary microcystin (-LR) for quart medaka

Dong-Fang Deng [a], Keke Zheng [b], Foo-Ching Teh [a], Peggy W. Lehman [c], Swee J. Teh [a,*]

[a] Center for Health and the Environment and Aquatic Toxicology Program, School of Veterinary Medicine, University of California, 1321 Haring Hall, One Shield Ave, Davis, CA 95616, USA
[b] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong Province 266071, China
[c] Department of Water Resources, Division of Environmental Services, Sacramento, CA 95691, USA

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ABSTRACT

This study was designed to estimate the toxic threshold of male and female fish to microcystins based on different biomarkers. Japanese medaka (*Oryzias latipes*) were fed dietary Microcystin-LR (0, 0.46, 0.85, 2.01 and 3.93 μg MC-LR/g dry diet for 8 weeks at 25 °C. The results revealed that dietary MC-LR inhibited growth at the end of 8 weeks. The survival of embryos and the RNA/DNA ratio of whole fish decreased significantly ($P < 0.05$) in fish fed 3.93 μg MC-LR/g dry diet. Heat shock protein (Hsp60) expression was induced in the liver of female and male fish fed diets containing ≥0.85 and 0.46 μg MC-LR/g diet, respectively. The activity of liver caspase 3/7 was significantly higher in female fish fed 3.93 μg MC-LR/g diet and in males fed 2.01 MC-LR μg/g dry diet than fish fed the control diet. The threshold for inhibition of liver protein phosphatase expression was lower in female (2.01 μg/g diet) than that in male fish (3.93 μg/g diet). Histopathological examination showed significant single-cell necrosis in female and male medaka fed diets containing 0.85 and 3.93 μg MC-LR/g diet, respectively. Based on different biomarkers, this study demonstrated that dietary MC-LR is toxic to Medaka and the effects are gender dependent.

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1. Introduction

Cyanobacteria (*Microcystis aeruginosa*) blooms are known to cause deleterious effects in aquatic ecosystems including zooplankton, fish, waterfowl, mammals, and humans. The most common and well-studied cyanotoxin is the hepatotoxin microcystin-LR (MC-LR), which has been on the rise in abundance and distribution in the upstream portion of the San Francisco Estuary since 1999 (Lehman et al., 2005). Of the 70+ isoforms of microcystins identified, MC-LR is the most studied, toxic and common (Zurawell et al., 2005). Although MC-LR specifically targets liver (Carmichael, 1995), it also impairs the function of other organs such as kidney, gills and the gastrointestinal tract (Rabergh et al., 1991; Kotak et al., 1996; Carbis et al., 1997). Several studies have reported the impact of MC-LR on the reproductive system in mice (Ding et al., 2006), rat (Li et al., 2008; Xiong et al., 2009), and fish (Baganz et al., 1998). Furthermore, field investigations on aquatic invertebrates and fish have strongly implicated the adverse effect of MC-LR on reproductive organs (Chen and Xie, 2005; Zhang et al., 2009).

MC-LR toxicity is the result of inhibition of phosphatase (PP1/PP2A) activity (Runnegar et al., 1993) and destruction of the cytoskeleton, which ultimately leads to cytotoxicity, interruption of cell division, and tumor-promoting activity (Carmichael, 1994; Humpage and Falconer, 1999; Fischer et al., 2000). Microcystin toxicity is also due to oxidative stress that causes apoptosis or necrosis depending on exposure concentration and duration (Ding and Ong, 2003; Li et al., 2005, 2007; Morena et al., 2005; Cazenave et al., 2006).

Most investigations on microcystin toxicity were based on aqueous (Tencalla et al., 1994), one time force-feeding (Tencalla and Dietrich, 1997), short term dietary exposure bioassays (Juhel et al., 2006) that determine the acute effects of microcystins on fish (Sun et al., 2008). Only

* Corresponding author. Fax: +1 530 752 7690.
E-mail address: sjteh@ucdavis.edu (S.J. Teh).

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limited information is available on the chronic dietary effect of microcystin on fish (Xie et al., 2004; Zhao et al., 2006) which was shown to be the major route of microcystin toxicity for fish in the natural environment (Zhang et al., 2009). In addition, there has been growing evidence to indicate that a suite of environmental chemicals, both anthropogenic and those occurring naturally, have the potential to alter endocrine-mediated sexual development resulting in disruption of gonadal sex differentiation and gametogenesis (Shutt, 1976; Bergeron et al., 1994; White et al., 1994; Kelce and Wilson, 1997; Gray et al., 2006). Medaka (*Oryzias latipes*) is a well-studied, highly-responsive fish model that has been used successfully to characterize acute and chronic toxicity in fish. Recent studies have also demonstrated that medaka is an appropriate model for studying toxic effects of cyanobacteria (Jacquet et al., 2004; Huynh-Delerme et al., 2005; Escoffiera et al., 2007; Mezhoud et al., 2008). To our knowledge, there is no information on gender effects of MC-LR on fish. The purpose of this study was to determine the dietary toxic threshold of MC-LR on male and female medaka based on integrated biomarkers. We hypothesized that 1) MC-LR affects reproduction performance in fish and 2) the sensitivity to the toxic effect of MC-LR is gender dependent.

2. Materials and methods

2.1. Experimental diets

Five test diets were prepared to contain graded levels of microcystin-LR (MC-LR), Dietary levels were: 0, 0.46, 0.85, 2.01 and 3.93 μg/g dry diet, respectively, and the levels of MC-LR were analyzed based on the method described by Hu et al. (2008). MC-LR (*M. aeruginosa*, $C_{49}H_{74}N_{10}O_{12}$) was purchased from EMD Biosciences Inc. (San Diego, CA, USA). The control diet was formulated without supplementation of MC-LR. The basal diet contained (g/kg): vitamin free casein, 310; wheat gluten, 150; dextrin, 272; egg albumin, 40; soy lecithin, 52; non nutritive bulk, 36; cod liver oil, 50; corn oil, 20; vitamin premix, 40; and mineral premix, 30. Except for the vitamin and mineral premixes, which were purchased from ICN (Biomedical, Inc., Irvine, CA), all other ingredients were obtained from U.S. Biochemical Corporation (Cleveland, OH, USA). The dry ingredients were thoroughly mixed before the oil was added. Double distilled water containing different concentrations of MC-LR (previously dissolved in methanol) was added to make wet dough. Pellets were prepared, freeze-dried and stored in the dark at −20 °C until use (Deng et al., 2008).

2.2. Dietary exposure of MC-LR

Embryos of Japanese medaka (*O. latipes*) were collected from our medaka culture system and separated by gender within 4 days post fertilization based on sex-linked coloration (Wada et al., 1998). After hatching (usually 8–10 days post-fertilization), larvae were cultured in a recirculation system with 20 fiberglass tanks (20 L per tank) and fed three times daily with the basal purified diet until used for the exposure study. Water flow-rate and temperature was 0.9 L/min and 25 ± 1 °C, respectively. Water quality including dissolved oxygen (8.3 mg L^{-1}), pH (7.8), water hardness (120 mg L^{-1}), and ammonium (not detectable) were monitored weekly.

The dietary exposure of MC-LR was conducted using 7-week old medaka. The initial body weight of fish was 82 ± 2 mg. Four tanks were randomly assigned to each dietary treatment with 2 tanks per gender and 100 fish per tank. Fish were fed twice per day (0900 and 1500 h) based on 5% of body weight daily. Water flow was stopped during feeding to prevent contamination of the recirculation system with dissolved MC-LR. The waste, uneaten feed and 50% of the water were siphoned from each tank 30 min after each feeding. To ensure that dissolved MC-LR from the diets did not contribute to any significant health effect to the fish, charcoal filters were changed weekly and 100% of the water in the recirculation system was replaced each day. Care, maintenance, handling, and tissue sampling of the fish followed the protocols approved by the University of California-Davis Animal Care and Use Committee.

2.3. Growth and reproduction

Fish were weighed at the end of 2, 4 and 8 weeks of feeding to estimate fish growth. In addition, at the end of 4 weeks of feeding, 30 females and 20 males fed the same dietary MC-LR concentration were mixed and allowed to breed to estimate reproductive performance. The fecundity (egg production per female) and survival of embryos were monitored each morning.

2.4. Sampling

At the end of 8 weeks, fish were killed by an overdose of MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA). Fish were weighed and measured for length, then separated into three groups. Group 1): 5 females and 5 males from each replicate tank were fixed in 10% neutral buffered formalin for histopathological examination by the method described by Teh et al. (2004). Group 2): 5 females and 5 males from each replicate tank were dissected to remove liver and ovary tissues. Liver and ovary tissues were weighed to estimate liver and ovarian somatic indices. Tissues were frozen in liquid nitrogen and stored at −80 °C until used for stress protein, protein phosphatase analysis and enzyme assay. Group 3): 5 females and 5 males from each replicate tank were frozen in liquid nitrogen and stored at −80 °C until pulverized with liquid nitrogen using a Freezer/Mill (SPEX SamplePrep, L.L.C., Metuchen, NJ, USA) and used for RNA/DNA analysis to estimate fish growth and recent feeding status.

2.5. Sample analysis

Fixed samples for histopathology were dehydrated in a graded ethanol series and embedded with both surgically cut sections face down in paraffin. Serial longitudinal sections (3 μm) were stained with hematoxylin and eosin (H&E), and lateral views of liver, kidney and gonads were screened for a variety of histopathological features and lesions. Livers were analyzed for lesions of glycogen depletion (GD), lipidosis (LIP), and single-cell necrosis

(SCN) and scored on an ordinal ranking system of 0 = none/minimal, 1 = mild, 2 = moderate, and 3 = severe using a BH-2 Olympus microscope as described by Teh et al. (2004). Briefly, Glycogen depletion (GD) is characterized by decreased size of hepatocytes, loss of the "lacy", irregular, and poorly demarcated cytoplasmic vacuolation typical of glycogen and increased cytoplasmic basophilia (i.e., blue coloration). Fatty vacuolar degeneration or lipidosis (LIP) is characterized by excess lipid appears as clear, round, and well demarcated cytoplasmic vacuoles. Single cell necrosis (SCN) is characterized by cells having eosinophilic (i.e., pink coloration) cytoplasm with nuclear pyknosis and karyorrhexis.

For stress protein and enzyme assays, frozen liver samples were extracted for protein according to the method described by Deng et al. (2009) using an ice-cold T-PER tissue protein extraction reagent (Pierce, Rockford, IL). Protein concentration of the supernatant was determined by the improved Lowry method (Bio-Rad, DC Protein Assay kit). Supernatant protein was used for analysis of stress protein, caspase and protein phosphatase activity.

Expression of Hsp60 and protein phosphatase in liver was analyzed by western block technique as described by Deng et al. (2009) except that 10% Tris–HCl precasted gels were used in the current study. The primary and second antibodies for PP1 and PP2A were purchased from Santa Cruz Biotechnology, Inc (CA, USA). The antibodies of stress proteins were purchased from Assay Designs Inc. (Ann Arbor, MI, and Santa Cruz Biotechnology, Inc, CA, USA). Equal amounts of protein (25 μg) were loaded onto 10% Tris–HCl precasted gels and separated by one-dimensional SDS-PAGE gel. Western blot and ECL (enhanced chemiluminescence) detection were performed by the methods of Hemre et al. (2004). Protein bands were quantified by a GS-710 imaging densitometer (Bio-Rad, Hercules, CA, USA). Protein standard (Assay Designs Inc., Ann Arbor, MI, and Santa Cruz Biotechnology, Inc, CA, USA) and molecular weight markers (Amersham Biosciences Corp, Piscataway, PA, USA) were loaded with samples in each gel to confirm the molecular mass of bands. The relative band density was calculated by comparing the band density of sample to that of standard.

Caspase-3/7 was determined by Apo-One Homogeneous caspase-3/7 Assay kit (Promega Corporation, Madison, WI, USA). Fluorescence activity was determined at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by a Spectra Max M2 micro plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The caspase activity was expressed as fluorescence/mg protein. Nucleic acids were measured by an ethidium bromide fluorometric technique (Caldarone et al., 2001) and quality control of analysis followed the same protocol as Deng et al. (2009).

2.6. Statistics

Data are presented as means ± standard errors and tested for homogeneity of variance before being analyzed by STATISTITC 6.0 software. All data were subjected to two-way analysis of variance to determine treatment and their interaction effects. Significant differences between the effect of dietary microcystin × gender were estimated by a Fisher LSD test ($P < 0.05$).

3. Results

3.1. Growth and reproduction performances

During the first 4 weeks of feeding, dietary microcystin did not affect fish growth or condition factor (CF), an index of fish fitness (Table 1). There was no difference in body weight (BW) between gender. The value of CF, however, was higher ($P < 0.05$) in female than in male fish after 4 weeks of feeding. At the end of 8 weeks of feeding, female fish fed the diet containing 0.85 μg MC-LR/g diet and male fish fed the 0.46 μg MC-LR/g diet had significantly lower BW than fish fed the control diet (Table 2). The BW and HSI were also higher in female fish than in male fish.

Dietary MC-LR did not show any adverse effect on gonadosomatic Index (GSI = 100 × gonad weight (g)/body weight (g)), fecundity or embryo weight (Table 3). Survival was significantly decreased in embryos collected from fish fed the 3.93 μg MC-LR/g diet. Gender differences in the RNA/DNA ratio were not observed in fish fed similar dietary treatments. However, female and male medaka fed the 3.93 and 2.01 μg MC-LR/g diet had significantly lower RNA/DNA ratios compared to control fish (Fig. 1).

3.2. Molecular biomarkers and histopathology

The levels of hsp60 were significantly induced in female and male medaka fed 0.85 and 0.46 μg MC-LR/g diet, respectively (Fig. 2). Dietary MC-LR did not affect the levels of hsp70 in liver (data not shown). Measurement of apoptosis, i.e., caspase 3/7 activities increased significantly in female fish fed the 3.93 μg MC-LR/g diet and in male fish fed ≥2.01 μg MC-LR/g diet (Fig. 3). The levels of PP1 in liver decreased significantly in females fed ≥2.01 μg MC-LR/g diets but no changes were observed in males. The PP2A levels in liver of female and male fish were both significantly inhibited when fed the 2.01 and 3.93 μg MC-LR/g diet, respectively. Histopathological examination showed that prevalence of lesions and the liver lesion score were generally increased with increased concentrations of dietary MC-LR (Tables 4–6). Female fish fed the diets containing 0.85 and 3.93 MC-LR showed significant higher lesion scores for single-cell necrosis compared to controls (Table 4). Male fish fed the diet containing 3.93 MC-LR had the highest lesion scores for lipidosis compared to that in fish fed the control diet (Table 5).

4. Discussion

Growth inhibition has been observed in different species of fish exposed to microcystin toxins. For example, depressed growth was observed in carp (*Cyprinus carpio* L.) after oral feeding of microcystis for 28 days at a dose of 50 μg microcystin/kg body weight (Li et al., 2004). The growth of brown trout (*Salmo trutta* L.) was retarded by exposure of fish to water contaminated with MC-LR (Bury et al., 1995). The current study reveals that the effect of dietary MC-LR is both dose and time dependent. Our results indicate that 4 weeks of dietary exposure was too short to impair growth of medaka. The lower BW and CF in medaka fed dietary MC-LR for 8 weeks may be the result of shifting

Table 1
Growth performance of medaka exposed to test diets for 2 and 4 weeks.

MC-LR (ug/g)	BW_2 (mg)		CF_2		BW_4 (mg)		CF_4	
	Female	Male	Female	Male	Female	Male	Female	Male
0	119.3 ± 2.6	143.0 ± 7.2	0.85 ± 0.01	0.88 ± 0.02	169.0 ± 7.3	168.2 ± 5.9	0.88 ± 0.04^{x}	0.80 ± 0.01^{y}
0.46	119.9 ± 9.1	133.1 ± 3.6	0.87 ± 0.00	0.84 ± 0.02	170.2 ± 13.8	165.9 ± 7.4	0.87 ± 0.02^{x}	0.80 ± 0.02^{y}
0.85	110.1 ± 6.4	125.6 ± 2.4	0.86 ± 0.01	0.86 ± 0.05	172.8 ± 9.3	167.8 ± 3.5	0.85 ± 0.02^{x}	0.77 ± 0.02^{y}
2.01	128.7 ± 1.6	138.7 ± 2.7	0.85 ± 0.01	0.86 ± 0.03	166.3 ± 10.4	164.1 ± 5.1	0.90 ± 0.01^{x}	0.86 ± 0.03^{y}
3.93	123.3 ± 5.2	136.2 ± 7.0	0.82 ± 0.00	0.80 ± 0.02	164.9 ± 7.0	159.3 ± 9.3	0.88 ± 0.01^{x}	0.81 ± 0.01^{y}

Data are presenting as Mean ± SE. Initial weight of medaka was 81–84 mg. BW_2 and BW_4: Body weight of week 2 and week 4, respectively; CF_2 and CF_4: Condition factor of week 2 and week 4, respectively. Letter x and y indicate significant difference between male and female fish fed the same diet ($P < 0.05$). Condition factor (CF) = 100 × body weight (g)/body length $(cm)^3$.

Table 2
Growth performance of medaka exposed to test diets for 8 weeks.

MC-LR (ug/g)	BW (mg)		CF		HSI (%)	
	Female	Male	Female	Male	Female	Male
0	290.7 ± 12.2^{a}	273.4 ± 12.7^{a}	$1.07 \pm 0.04^{a,x}$	$0.99 \pm 0.05^{a,y}$	2.78 ± 0.18^{x}	1.63 ± 0.13^{y}
0.46	271.9 ± 12.8^{ab}	245.9 ± 7.3^{b}	$0.95 \pm 0.02^{b,x}$	$0.85 \pm 0.01^{b,y}$	2.96 ± 0.18^{x}	1.52 ± 0.12^{y}
0.85	256.2 ± 11.3^{b}	232.5 ± 7.9^{b}	$0.93 \pm 0.02^{b,x}$	$0.84 \pm 0.02^{b,y}$	2.95 ± 0.19^{x}	1.64 ± 0.08^{y}
2.01	271.8 ± 9.8^{ab}	248.9 ± 5.7^{ab}	$0.97 \pm 0.01^{b,x}$	$0.84 \pm 0.03^{b,y}$	2.73 ± 0.22^{x}	1.76 ± 0.10^{y}
3.93	257.8 ± 9.3^{b}	248.5 ± 6.6^{ab}	$0.92 \pm 0.02^{b,x}$	$0.81 \pm 0.01^{b,y}$	2.60 ± 0.17^{x}	1.63 ± 0.12^{y}

Data are presenting as Mean ± SE. BL: Body length; BW: Body weight; CF: Condition factor; HSI: Hepatosomatic index. Different letters indicate significantly difference among different dietary treatments ($P < 0.05$). Different letters within the same column (a, b) indicate significant difference among dietary treatments and the letters within the same row (x, y) indicate significant difference between gender fed the same diet ($P < 0.05$). Hepatosomatic Index (HSI) = 100 × liver weight/body weight (g).

Table 3
Reproduction performance of medaka exposed to test diets for 8 weeks.

MC-LR (ug/g)	Ovarian ratio (%)	Fecundity (egg number/female)	Embryo weight (mg)	Survival of embryo (%)
0	6.31 ± 0.38	4.1 ± 0.3	1.12 ± 0.01	62.3 ± 2.0[a]
0.46	5.18 ± 0.40	3.5 ± 0.3	1.13 ± 0.01	62.7 ± 1.8[a]
0.85	6.21 ± 0.36	3.6 ± 0.1	1.11 ± 0.01	68.1 ± 2.4[a]
2.01	5.63 ± 0.37	3.9 ± 0.5	1.14 ± 0.01	63.8 ± 2.8[a]
3.93	7.04 ± 0.98	3.7 ± 0.2	1.10 ± 0.01	54.9 ± 2.6[b]

Data are presenting as Mean ± SE. Different letters (a, b) indicate significantly difference among different dietary treatments ($P < 0.05$).

energy in the fish from supporting growth to handling chronic stress due to the toxin.

Dietary MC-LR at the level of 3.93 μg MC-LR/g diet significantly impaired reproductive performance of medaka based on embryo survival in this study. The low survival of embryos is likely due to the effect of MC-LR on embryo quality, which may have affected egg or sperm quality and fertilization process or other unknown factors that were not investigated in this study. Jacquet et al. (2004) found that medaka embryos had low survival and earlier hatching when exposed to MC-LR by microinjection. Oberemm et al. (1997) also found that MC-LR in water (5–50 μg/L) decreased embryo development and retarded larval growth of zebra fish. Similarly, exposure of southern catfish fertilized eggs to crude extracts of MCs (10–100 mg MC-LR eq L^{-1}) delayed egg and larval development, reduced hatching rate, increased malformation rate and hepatocytes damage in larvae (Zhang et al., 2008). Although the mechanism of MC-LR on reproduction is not well understood, oxidative stress as well as an increased energy demand for detoxification in adult fish or the trophic transfer of MC-LR to the embryo may cause the deleterious effects of microcystins on fish earlier life stages when organogenesis is not completed (Wiegand et al., 1999). The current study presents strong evidence that dietary *Microcystis* reduces fish reproduction. This effect of MC-LR on embryo survival or hatching could be a contributing factor to the decline of pelagic fish populations exposed to microcystins through their food web in the San Francisco estuary.

The RNA/DNA ratio, an indicator of recent growth or nutritional condition of fish (Clemmesen et al., 1997; Buckley et al., 2004), decreased in medaka fed ≥2.01 μg MC-LR/g diets suggests that MC-LR inhibited protein synthesis and thus growth. This further supports our assumption that decreased food intake or shifting of energy from growth to toxin stress and is in agreement with the reduction in growth by dietary MC-LR as discussed above. The lower threshold of MC-LR inhibiting protein synthesis (RNA/DNA ratio) in females than males indicated that the growth of females is more sensitive than males to the effects of MC-LR.

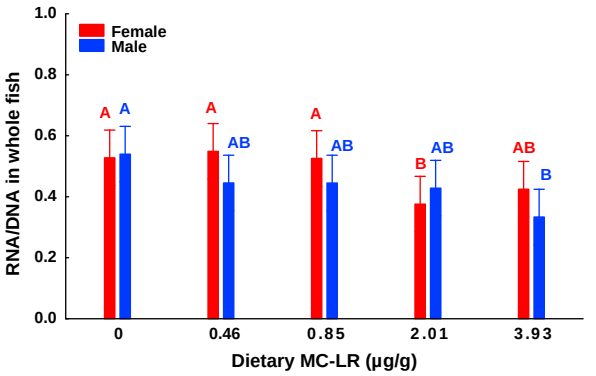

Fig. 1. The ratios of RNA/DNA in whole fish fed different levels of dietary MC-LR for 8 weeks. Data are presented as Mean ± SE. Different letters indicate significant difference among dietary treatments within the same gender ($P < 0.05$).

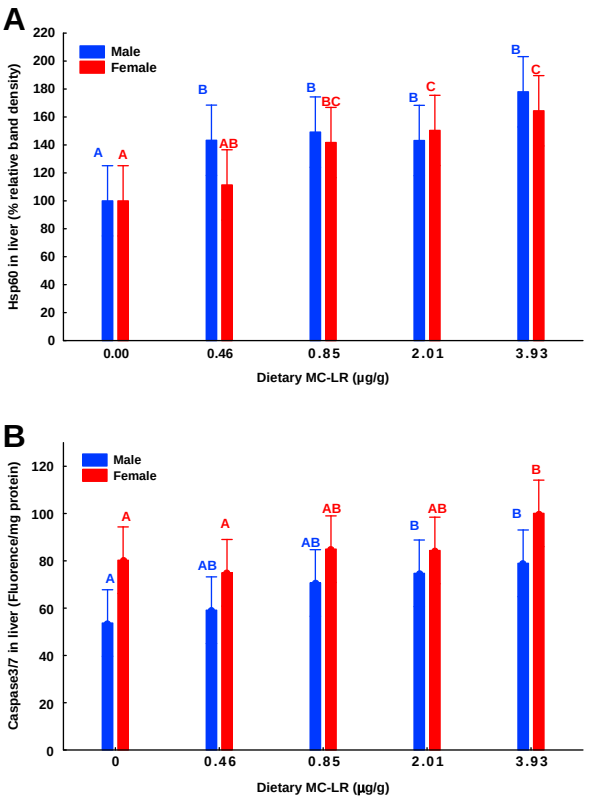

Fig. 2. The Hsp60 expression (A) and activity of caspase 3/7 (B) in liver of medaka fed different levels of dietary MC-LR for 8 weeks. Data are presented as Mean ± SE. Different letters indicate significant difference among dietary treatments within the same gender ($P < 0.05$).

Heat shock proteins (Hsp), also called stress protein, are a group of structurally conserved proteins present at a relative low level under normal physiological conditions (Basu et al., 2002). The level of Hsp, however, can be induced via a wide variety of stressors such as temperature change (Deng et al., 2009), contaminant exposure (Sanders, 1993; Werner and Nagel, 1997) or feeding (Cara et al., 2005; Deng et al., 2009). Heat shock protein is also involved in apoptotic processes through their role as chaperones. Hsp60 is mainly localized in the mitochondrial matrix (Werner and Nagel, 1997). The increased level of Hsp60 in Medaka liver tissue for this study suggests that fish turn on their earlier defense mechanism when exposed to diets containing MC-LR as low as 0.46–0.85 μg MC-LR/g diet. Increasing levels of dietary MC-LR, however, may surpass the defense capacity of stress protein to deal with the folding or degradation proteins and eventually damage the

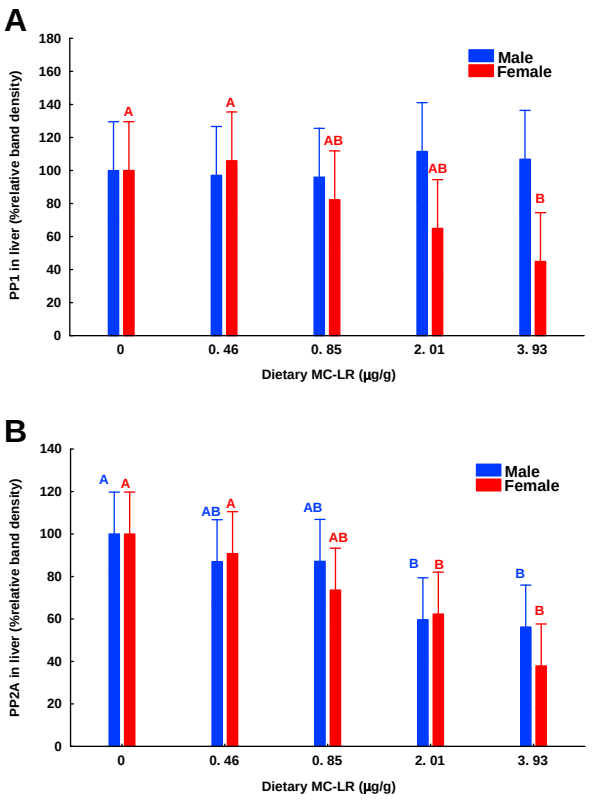

Fig. 3. The protein phosphatase PP1 (A) and PP2A (B) expression in liver of medaka fed different levels of dietary MC-LR for 8 weeks. Data are presented as Mean ± SE. Different letters indicate significant difference among dietary treatments with the same gender ($P < 0.05$).

Table 4
Summary of histopathology scores in female medaka fed different diets.

Dietary MC-LR (μg/g)	Parameters	Fish number of different lesion score				Average score	Standard error
		0	1	2	3		
0	GD	11	4	3	2	0.80[a]	0.46
0.46	GD	10	4	5	1	0.85[a]	0.40
0.85	GD	8	3	8	1	1.10[a]	0.33
2.01	GD	11	2	2	5	1.20[a]	0.69
3.93	GD	7	2	4	7	1.55[a]	0.57
0	SCN	14	6	0	0	0.25[a]	0.05
0.46	SCN	12	8	0	0	0.40[ab]	0.08
0.85	SCN	7	13	0	0	0.65[b]	0.10
2.01	SCN	12	8	0	0	0.40[ab]	0.14
3.93	SCN	9	10	1	0	0.60[b]	0.14
0	LIP	14	5	1	0	0.30[a]	0.24
0.46	LIP	13	5	2	0	0.45[a]	0.17
0.85	LIP	12	8	0	0	0.40[a]	0.14
2.01	LIP	14	4	1	1	0.45[a]	0.26
3.93	LIP	9	5	2	4	0.90[a]	0.42

GD: glycogen depletion; SCN: single-cell necrosis or piecemeal necrosis; Lip: lipidosis or hepatocellular vacuolation. Scores represent ordinal effects: 0 = non/minimal. 1 = mild, 2 = moderate, and 3 = severe. Different letters indicate significant difference among dietary treatments ($P < 0.05$). Twenty fish from each dietary treatment were evaluated for histopathology.

Table 5
Summary of histopathology scores in male medaka fed different diets.

Dietary MC-LR (μg/g)	Parameters	Fish number of different lesion score				Average score	Standard error
		0	1	2	3		
0	GD	14	5	1	0	0.35[a]	0.17
0.46	GD	12	6	2	0	0.50[a]	0.13
0.85	GD	7	8	3	2	1.00[a]	0.38
2.01	GD	8	4	6	2	1.10[a]	0.48
3.93	GD	5	2	9	4	1.50[a]	0.53
0	SCN	17	3	0	0	0.15[a]	0.10
0.46	SCN	19	1	0	0	0.05[a]	0.05
0.85	SCN	14	6	0	0	0.30[a]	0.13
2.01	SCN	18	2	0	0	0.10[a]	0.10
3.93	SCN	12	8	0	0	0.40[a]	0.22
0	LIP	18	2	0	0	0.10[a]	0.06
0.46	LIP	14	4	2	0	0.40[ab]	0.14
0.85	LIP	9	8	1	2	0.80[ab]	0.18
2.01	LIP	12	8	0	0	0.35[ab]	0.10
3.93	LIP	10	3	5	2	0.95[b]	0.35

GD: glycogen depletion; SCN: single-cell necrosis or piecemeal necrosis; Lip: lipidosis or hepatocellular vacuolation. Scores represent ordinal effects: 0 = non/minimal. 1 = mild, 2 = moderate, and 3 = severe. Different letters indicate significant difference among dietary treatments ($P < 0.05$). Twenty fish from each dietary treatment were evaluated for histopathology.

cell. As a consequence, more defense or protection mechanisms will be needed to prevent further damage. Apoptosis is a physiological process involving caspase as one of the executers that eliminate damaged or unwanted DNA from the cell. This prevents cells from further deleterious effects damaged proteins or cells. Therefore, the increased caspase activity in fish fed high dietary MC-LR (2.01–3.93 μg/g diet) suggests that an apoptosis pathway was involved in the protection of medaka from toxic effects at high dietary concentrations of MC-LR. The threshold based on Hsp60 expression and caspase activity was lower for males than females, suggesting that the defense mechanism in male medaka is more responsive to dietary MC-LR than in females. This sensitive or efficient protection mechanism may have protected the male fish from further harmful effects by the MC-LR toxin. This is further supported by the less significant inhibition of protein phosphatase expression (PP1 and PP2A) in males than in females. The threshold based on protein phosphatase inhibition was generally lower in females (2.01–3.93 μg/g diet) than in males (≥3.93 μg/g diet). The current study also

Table 6
The toxic threshold of dietary MC-LR for medaka based on different biomarkers.

Parameters	Effective concentration (μg MC-LR/g diet)	
	Female	Male
Body weight and CF	0.46–0.85	0.46
Stress protein	0.85	0.46
RNA/DNA	2.01	3.93
Embryo survival	3.93	
Apoptosis (caspase3/7)	3.93	2.01
Protein phosphatase	2.01–3.93	2.01
Histopathology	0.85–3.93	3.93

demonstrated that MC-LR was not only inhibiting the activity of protein phosphatase as have been found in previous study (Runnegar et al., 1993 but also decreasing the enzyme levels in liver in this study. The long term exposure of high dose of MC-LR may have resulted in necrosis of cell and thus protein (enzyme) synthesis was decreased. The mechanism for MC-LR inhibiting the enzyme expression, however, is not studied in this experiment and will be needed for future research.

The dietary MC-LR (0.85 μg/g diet) led to single-cell necrosis in the liver of female but not male Medaka also suggested that females are more sensitive to this toxin. Gender difference in tumor incidence has been observed in medaka exposed to diethylnitrosamine, with higher incidence and faster development in foci and tumors in females than in males (Teh and Hinton, 1998). The mechanism is not clear but steroid hormones may play an important role in these differences between genders.

In summary, our results demonstrated that the toxic threshold of dietary MC-LR for medaka is different between males and females (Table 6). Male medaka appears to have a more sensitive defense mechanism than female medaka but females are more sensitive to the toxic effect of MC-LR. As a consequence, the effective toxic thresholds such as RNA/DNA ratio, inhibition of protein phosphatase and occurrence of cell necrosis were lower in mature females (2.01 μg MC-LR/g diet) than in males (3.93 μg MC-LR/g diet). The decreased survival of embryos by dietary MC-LR suggests a possible reason for the reported decline in fish population, especially pelagic species of fish, in habitats where toxic algal blooms naturally occur.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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